PROGRESS REPORT

SPECIFIC AIMS

Aim 1. Evaluate Zatolmilast pharmacotherapy of cognitive sequalae of ARSACS***.*** Zatolmilast is a negative allosteric modulator of PDE4D that has recently shown positive outcomes in a phase 2 clinical trial adolescent male with fragile X syndrome (FXS, (Berry-Kravis, Harnett et al. 2021)). Recently disseminated preliminary results from two phase 3 trials are even more encouraging (NPR). Because of our preclinical efforts, the same drug has entered, in May 2025, a phase 2 clinical trial for the treatment of the neurodevelopmental disorder Jordan's Syndrome (JS, Shionogi press release). Leveraging our expertise in preclinical development, we propose to test the effectiveness of Zatolmilast for treatment of both motor- and cognitive phenotypes of *Sacs* KO mice.

We faced delays some obtaining Zatolmilast from the manufacturer and co-sponsor of the JS clinical trial. After several months without progress, we have finally completed an NDA and are in the process of completing an MTA with Shionogi, who bought up Tetra Therapeuticals, the maker of Zatolmilast (BPN14770). We expect to be in receipt of the requested 2 grams of Zatolmilast (BPN14770) soon. We opted to wait for pharmaceutical-grade drug rather than purchasing BPN14770 from MedchemExpress.

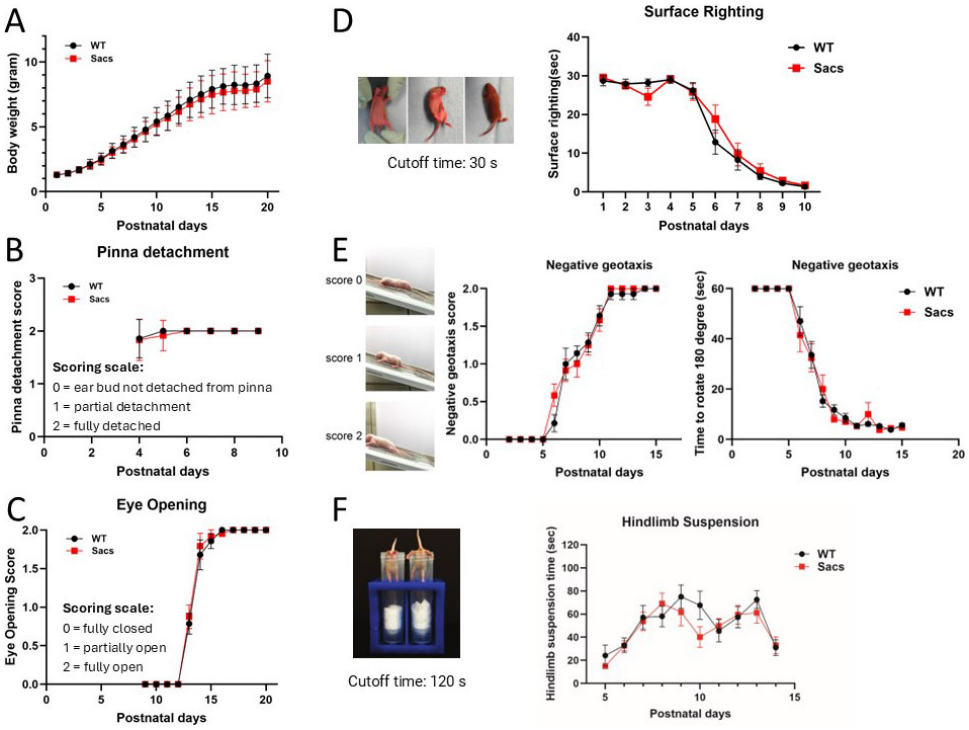

Figure 1. ***Sacs KO mice meet major developmental milestones. A,*** Sacs KO and wild-type littermates gain equivalent weight during weaning. ***B***, pinna detachment is not delayed in *Sacs* KO pups. ***C,*** no delay in eye opening is *Sacs* KO pups. ***D***, the *Sacs* KO does not affect development of the righting reflex. ***E***, development of the negative geotaxis reflex (quantified as 0-2 score (left) and time to 180^0 rotation (right)) is not compromised by loss of *Sacs*. ***F***, no effect of the Sacs KO in the hindlimb suspension test.

In the meantime, we decided to comprehensively characterize early postnatal development of global *Sacs* KO mice, a period that corresponds to the third human trimester. We did this because Zatolmilast is currently in clinical trials for two neurodevelopmental disorders. We tested 19 pups of homozygous *Sacs* KO mice and 23 wild-type littermate controls of roughly equal sex distribution. We identified no sex differences and therefore show data from male and female mice combined in **Fig. 1**. During weaning, Sacs KO mice gained weight (**Fig. 1A**), and reached morphological (**Fig. 1B,C**) milestones as soon as wild-type littermates. As well, surface righting and negative geotaxis reflexes developed similarly (**Fig. 1D,E**). Muscle strength as measure by the hindlimb suspension test was no different between the two pup genotypes (**Fig. 1F**). We conclude that ARSACS, at least as modeled in *Sacs* KO mice, is not an early onset neurodevelopmental disorder.

We also began longitudinally testing of cohorts of germline Sacs KO mice in order to determine the time course of motor vs cognitive phenotypes. An additional rationale was to ascertain when to best start pharmacological or gene therapy. We had previously shown that two separate cohorts of Sacs KO mice of 6-8 mo age displayed only chance-level performance in the 2-trial Y-maze test of short-term memory. We just concluded tests with mice of both sexes at 3 month of age. We detected no significant differences in weight between homozygous *Sacs* KO mice and *Sacs*$^{+/+}$ mice (**Fig. 2A**), which removes a potential confound of behavioral, especially motor tests. Highly significant impairments in latency to cross were detected with both the 12 mm and 6 mm balance beam (**Fig. 2A**). There were no sex difference in this and subsequent assays, so data for male and female mice were combined. Accelerating RotaRod tests confirmed that the Sacs KO does not impair motor-coordination at this early (3 mo) age (no difference in latency to fall at day 1, **Fig. 2C**) (Lariviere, Gaudet et al.

2015). However, we observed that the performance of wild-type mice improved over time, whereas Sacs KO mice showed improvements over day 1 only on the third day (**Fig. 2C**). These data indicate that loss of Sacsin impairs motor learning in early adulthood. We next assessed short term learning and memory using the two-trial Y-maze (principle in **Fig. 2D**). Both genotypes performed equally well (**Fig. 2E**). In aggregate, these data suggest that the cognitive deficits older Sacs KO mice exhibit (Chen, Merrill et al. 2024) may not be due to cerebellar degeneration/dysfunction. We will examine this further using our conditional *Sacs* KO mice (Aim 3).

Aim 2. Explore gene therapy of ARSACS utilizing high payload adenoviral delivery of full-length human Sacsin. In collaboration with the Viral Vector Core at the U. Iowa (Montesinos, Chen et al. 2011, Montesinos, Satterfield et al. 2016), we propose to generate hdAd expressing full-length human Sacsin (13470 bp) with an N-terminal EGFP tag. In the long term, we will use this virus to a) rescue phenotypes in *Sacs* KO mice, and b) identify in vivo Sacsin interacting protein by affinity-isolation using GFP nanobodies and LC-MS^2. Initially, we will optimize stereotaxic delivery of the virus to the cerebellum (volume and titer) and examine transduction efficiency (GFP fluorescence) and potential cytotoxicity (cell loss, edema) *post hoc*.

We made major and exciting progress on this aim that suggests the feasibility of ARSACS gene therapy. As proof-of-concept, we generated the proposed, proprietary high-capacity *Sacs* gene delivery vector with changes proposed in the last grant application (CAG instead of synapsin promoter, GFP instead of V5 tag). In contrast to adenovirus, hdAd is "gutted" (replication-incompetent), so safe for gene therapy. The EGFP coding sequence was inserted between the poorly conserved exons 1 and 2 of the human reference transcript and before the Ubl domain. To verify expression, we infected HeLa cells and imaged intrinsic GFP fluorescence by structured illumination microscopy. We detected diffuse cytoplasmic localization of GFP-Sacsin. The protein also exhibited punctate localization especially around nuclei (**Fig. 3A**). We did not observe colocalization with dynamin-related protein 1 (Drp1) or mitochondria under these overexpression conditions (data not shown).

Next, we stereotaxically injected hdAd GFP-Sacsin into the cerebellum of a *Sacs* KO mouse, perfused the mouse after 3 weeks, and imaged the cerebellum by confocal microscopy with and without enhancing intrinsic fluorescence by GFP immunofluorescence. Non-enhanced, intrinsic GFP fluorescence revealed clusters of infected Purkinje

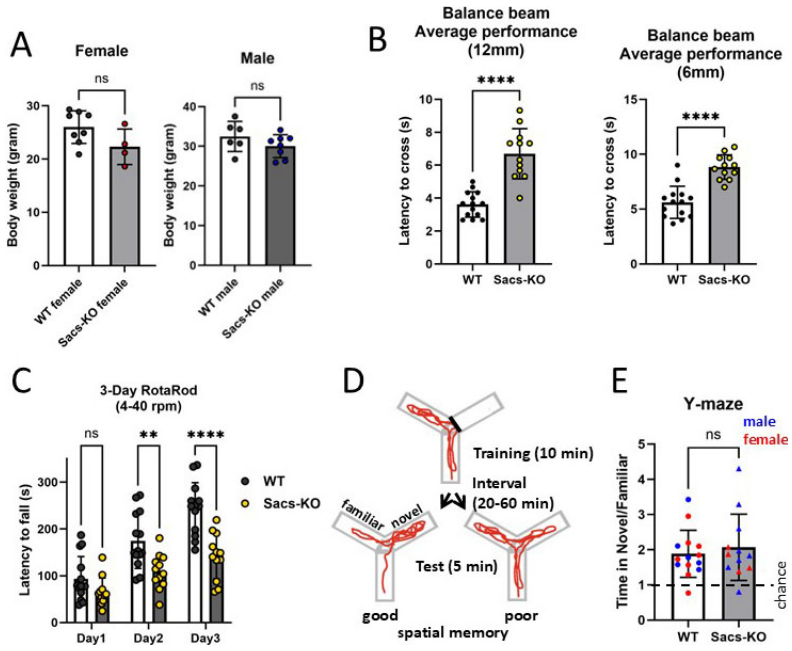

Figure 2. ***At 3 mo of age, Sacs KO mice display motor, but not yet cognitive deficits. A,*** *Sacs* KO and wild-type littermates weight about the same at 3 months. Weight is a factor in both RotaRod and balance beam tests. ***B***, *Sacs* KO mice take longer to cross a 12 mm (left) and 6 mm (right) wide beam. ***C***, *Sacs* KO mice display deficits in motor learning (latency to fall over time) but not motor coordination (latency to fall at day 1). ***D***, principle of the 2-trial Y-maze assay of short-term memory. ***E***, 3 mo old *Sacs* KO mice perform like wild-type mice in the 2-trial Y-maze. **,p<0.01;****,p<0.0001 by Students t-test or two-way ANOVA with Tukey post hoc test.

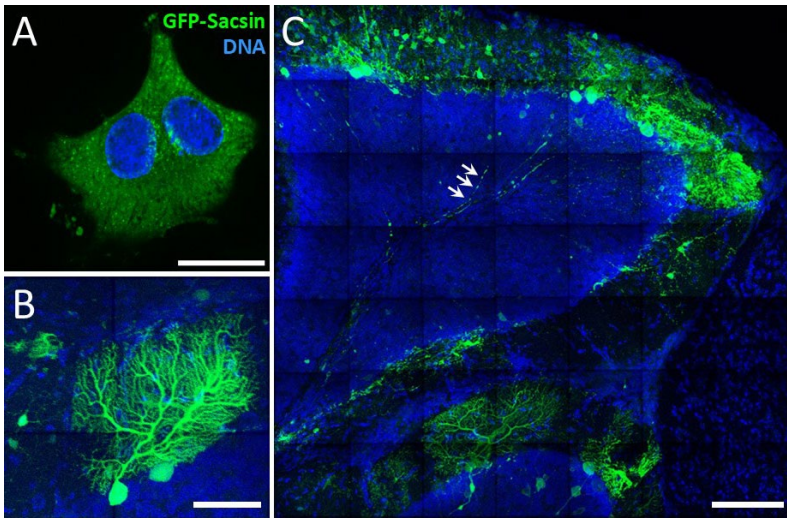

Figure 3. ***Efficient gene delivery of full-length GFP-Sacsin with a high-capacity adenoviral vector. A,*** GFP-Sacsin displays cytosolic localization with puncta enriched in the peri-nuclear region of HeLa cells (scale bar = 25 μm). ***B***, two Purkinje neurons and one presumptive stellate neuron transduced with GFP-Sacsin and visualized by intrinsic fluorescence (scale bar = 50 μm). ***C***, stitched Z-projection of 10 μm confocal stack of a cerebellar lobule. Intrinsic GFP-Sacsin fluorescence was enhanced by GFP immunofluorescence. Note that GFP-Sacsin is confined to the molecular layer. Expression in the granule cell layer is confined to Purkinje neuron axons (arrows); scale bar = 100 μm.

cell somata and dendritic arbors as well as neurons in the molecular layer, likely stellate cells (**Fig. 3B**). Cerebellar sections with labeling enhanced by GFP immunofluorescence displayed remarkably widespread GFP-Sacsin expression (**Fig. 3C**) given that we only performed a single virus injection. Still, expression was largely confined to the molecular layer, with only Purkinje neuron axons labeled in the granule cell layer (arrows in **Fig. 3C**).

Aim 3. ***Develop a conditional Sacs KO mouse model.*** Much has been learned from the germline (global) KO of the *Sacs* gene in mice. However, Sacsin is expressed during development and in all brain regions. For instance, as recently reported by us (Chen, Merrill et al. 2024) and extended to young-adult heterozygous mice (Fig. 1b in 2024 application), *Sacs* KO mice display robust deficits in learning and memory, brain functions not commonly associated with the cerebellum. As well, while Purkinje cell (PC) death is a convenient readout for cerebellar dysfunction and degeneration, it is unclear whether Sacsin loss in PCs per se drives motor pathology. Answering these and many other important questions (e.g. time window for intervention) requires a conditional *Sacs* KO model. For instance, crossing Pcp2-cre driver mice with cKO *Sacs* mice will delete the gene selectively in PCs, thus answering whether this is sufficient for ARSACS pathology. Collaborating with the Genome Editing Core at the U Iowa, the PI has extensive experience with the design and analysis of conditional mouse models (shown for the inducible *Ppp2r5d* E420K knock-in mouse model of Jordan's Syndrome in Fig. 2 of the 2024 application).

After implantation of targeting vector-injected zygotes into 768 pseudo-pregnant females over the span of 15 months, our Genome Editing Core obtained one *Sacs* cKO founder mouse in late 2024. After expanding the colony, we started breeding them with two well-characterized Cre driver lines. The Pcp2-Cre line leads to Purkinje cell-specific deletion of *Sacs*, whereas Emx1-Cre deletes the gene in all glutamatergic neurons of the cortex and hippocampus. Both crosses are now at the second generation. Because Emx1-Cre mice can be maintained as homozygotes, we are only one generation away from being able to set up crosses that will yield experimental animals and their controls. Since only heterozygous Pcp2-Cre mice can be maintained, it will take two generations to reach that stage. Justin Wolters is eagerly awaiting the Pcp2-Cre/cKO line and we hope to ship breeders to Madison, WI later this year.

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